Pest Manag Sci 58:769–774 (online: 2002)
DOI: 10.1002/ps.528

Group effects on insecticide toxicity in workers of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki†

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Abstract: ***Coptotermes formosanus*** **workers were treated topically with insecticide and subsequently held individually or in groups to examine possible effects on insecticide toxicity. Chlorpyrifos, cypermethrin and chlordane toxicities were 1.4-, 1.5-, and 1.3-fold greater, respectively, among workers held in groups compared with those held individually after insecticide treatment. Experiments were conducted to examine how enhanced toxicity occurred among termites held in groups after topical insecticide treatment. When workers were treated topically with chlordane and immediately placed with untreated workers, significantly greater numbers of untreated workers were killed compared with controls at all ratios examined (insecticide-treated:untreated). These data indicated that workers treated topically with insecticide were capable of somehow transferring a lethal dose of insecticide to untreated workers confined in the vial. Chlordane was recovered from untreated workers which had been confined with chlordane-treated workers; significantly higher quantities of chlordane were recovered from dead workers exposed to chlordane-treated workers compared with surviving workers exposed to chlordane-treated workers. Possible mechanisms of insecticide transfer from insecticide-treated to untreated termites are discussed.**
Published in 2002 for SCI by John Wiley & Sons, Ltd.

Keywords: insecticide toxicity; bioassay; trophallaxis; termites; chlordane; cypermethrin; chlorpyrifos; insecticide transfer; *Coptotermes formosanus*

1 INTRODUCTION

The exchange of stomodeal and/or proctodeal material between individuals of eusocial insect colonies, trophallaxis,[1] has been effectively exploited in Hymenoptera[2] and, recently, Isoptera[3] for the purpose of disseminating a slow-acting toxicant throughout the colony. If the toxicant is sufficiently slow killing, non-repellent, and freely exchanged among the worker caste,[4–6] then the colony may be eliminated either by directly or indirectly killing the reproductive(s) and all potential reproductives. Toxicant introduction has been typically achieved through the use of a spiked, palatable bait matrix.[7] However, among termites, successful deployment of a toxicant also has been reported by topically treating captured workers with a dust or transmissible resin coating and subsequently releasing the treated insects back into the colony.[8,9]

Despite awareness of trophallaxis behavior in termites, insecticide toxicity bioassays in termites have usually been conducted using a group of workers held together in a confined space.[10–13] We hypothesized that the practice of holding insecticide-treated worker termites in a group would increase the apparent toxicity of the insecticide, possibly through trophallaxis and/or grooming exchange. Furthermore, we were interested in examining the conditions that facilitate the dispersal of insecticide in Formosan subterranean termite workers held together in groups.

2 EXPERIMENTAL

2.1 Chemicals

Technical grade chlordane (mixture of isomers), cypermethrin (50% *cis*/48% *trans*) and chlorpyrifos were purchased from ChemService (West Chester, PA). Nile Blue A dye was purchased from Aldrich Chemical Co (Milwaukee, WI).

2.2 Termites

Formosan subterranean termites, *Coptotermes formosanus* Shiraki, were collected from field colonies in City Park, New Orleans, LA. Seven-inch round valve boxes (Part No 107BCH, NDS Inc, Lindsay, CA) were placed in the ground containing rolled corrugated

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(*Received 23 August 2001; revised version received 15 March 2002; accepted 4 April 2002*)

cardboard. The rolls of cardboard were collected twice weekly and brought back to the laboratory where the termites were separated from the debris. The termites were maintained in the laboratory at room temperature in hard plastic containers (Part No 079-C, Pioneer Plastics Inc, Dixon, KY) on moistened southern yellow pine blocks and used within seven days of collection.

2.3 Insecticide bioassays

A plastic weight boat with 10 workers was placed on crushed ice for 1 min. Termites were treated topically with insecticide in acetone (0.5 μl) applied to the abdomen. Five concentrations causing >0% and <100% mortality were used for each experiment which was replicated three times. The insecticide-treated termites were then immediately placed either as a group (10 termites) into a 20-ml glass scintillation vial containing a piece of filter paper (144 mm^2; Whatman No 1) moistened with deionized water (27 μl) or individually into wells of a 96-well, flat-bottomed, plastic microplate (Corning Inc, Corning, NY) with a 16 mm^2 piece of filter paper moistened with deionized water (3 μl). In both cases, a moistened tissue wiper was placed on top of the openings of the vials and microplate wells. The microplates and vials were then placed into a Rubbermaid plastic container (33 cm long × 12 cm tall × 21 cm wide) with deionized-water-moistened paper towels lining the bottom. The treated termites were placed into an incubator at 26 °C. Mortality was scored as an absence of movement for cypermethrin- and chlorpyrifos-treated termites (24-h endpoint). However, for the comparatively slower-acting chlordane, mortality was scored as the inability of a termite to right itself within 10 s after being flipped onto its dorsum. Preliminary experiments verified that termites meeting this criterion did expire (no movement) within 12 h (36 h after treatment). These observations were made using a Nikon, type 102, stereoscope set at 10× power.

2.4 Insecticide exchange in a group

To examine causes responsible for enhanced insecticide toxicity in a group, a mixture of insecticide-treated and untreated termites were placed together under different conditions. Termites were marked by allowing them to feed for 72 h on filter papers dyed with Nile Blue A.[14,15] A 9-cm diameter filter paper (Whatman No 1) was treated with an aqueous solution of Nile Blue A (2.5 g kg^{-1}; 1.5 ml). The filter paper disks were allowed to air-dry completely before use. When staining, 300 to 500 termites were placed into a Petri dish (150 mm × 15 mm) containing two dye-treated filter paper discs each moistened with deionized water (1 ml).

The first series of experiments examined mortality among a mixture of chlordane-treated and -untreated termite workers. Chlordane was chosen to examine the group toxicity effect because it is not repellent to termites[10] and chemical detection is easily accomplished. Furthermore, the termites exhibited the same responses (ie more susceptible in groups) to all of the insecticides examined. In all cases, Nile Blue A termites received the desired treatment so that insecticide-treated and untreated termites could be discerned in a mixture of the two groups. A series of Nile Blue A-dyed termites (one to nine) was placed with a series of untreated termites so that a sum of ten termites per vial was achieved (ie one untreated with nine treated, two untreated with eight treated, etc.). Dyed termites were treated with 0.4 μg of chlordane per termite (LD_{99} derived from bioassays of individually held termites) and placed immediately, or after death (48 h), with untreated termites, or were killed by having their head crushed by a pair of forceps. Corresponding control groups included a positive control (dyed termites treated with 0.4 μg of chlordane, LD_{99}), negative control (0.5 μl acetone treatment of dyed termites), and termites killed without the use of insecticide. Treated and untreated termites were placed together in a glass scintillation vial with moistened filter paper (144 mm^2), placed in an incubator at 26 °C and mortality measured in both groups at 24 h.

To examine the effect of insecticide exchange on toxicity more directly, a mixture of five workers dyed with Nile Blue A and treated with chlordane (0, 0.13 [LD_{25}], 0.16 [LD_{50}], 0.21 [LD_{75}], and 0.4 μg per termite [LD_{99}]) and five untreated workers were placed together in a vial as described above. Lethal dose values were derived from bioassays of individually held termites. Termites were held in an incubator at 26 °C and mortality among both chlordane-treated and untreated termites was assessed at 24 h. In addition, chlordane was extracted from the untreated surviving and dead termites of each treatment (LD_{25} through LD_{99}). Chlordane was extracted by first adding deionized water (0.5 ml) to a glass reaction vessel (10 × 3 cm^2) containing the termites. The termites were then macerated with a serrated Teflon pestle for about 1 min. Hexane (4 ml) was added to the reaction vessel, which was then vortexed at high speed for 2 min. The reaction vessel was centrifuged at 500 *g* for 3 min and an aliquot of the organic phase was removed and dried over anydrous sodium sulfate. The sample was analyzed by gas chromatography on a Hewlett-Packard model 5890 gas chromatograph (Avondale, PA) equipped with a splitless injector and an electron-capture detector. A 15-m DB608 capillary column (J&W Scientific, Folsom, CA) was used for all analyses. Injector, detector and column temperatures were set at 250, 300 and 210 °C, respectively. Recovery of chlordane from controls was 91.4 (±4.9)%.

2.5 Statistical analysis

Bioassay data were analyzed by probit analysis using insecticide dose as the independent variable.[16,17] Significant differences were determined by non-overlap of 95% fiducial limits. Trophallaxis experiments

Table 1. Lethal dose values for *Coptotermes formosanus* workers topically treated with insecticide and subsequently held in groups or as individuals

Insecticide	*Holding method*[a]	n	χ^2	df	*Slope (±SE)*	*LD_{50} (95% CI) (μg g^{-1} body weight)*
Chlorpyrifos	Individual	180	1.5	4	10.2 (±1.3)	5.61 (5.34–5.90)
Chlorpyrifos	Group	190	6.1	4	10.7 (±1.7)	4.0 (3.69–4.24)
Cypermethrin	Individual	150	3.4	3	7.3 (±1.3)	1.13 (1.05–1.28)
Cypermethrin	Group	150	3.9	3	7.6 (±1.1)	0.74 (0.68–0.80)
Chlordane	Individual	150	3.5	3	6.1 (±0.8)	59.3 (54.0–65.6)
Chlordane	Group	150	3.7	3	8.8 (±1.2)	45.5 (42.0–49.3)

[a] Termites were held individually in a flat-bottomed microplate well or together as a group of 10 (corresponding to each insecticide dose) in a 20-ml glass scintillation vial.

with different ratios of chlordane-treated termites were compared non-parametrically with untreated controls using the Mann–Whitney test.[17,18] The quantity of chlordane recovered from untreated surviving and dead termites was compared by Student's *t*-test.

3 RESULTS AND DISCUSSION

Toxicities (measured at the LD_{50}) of chlorpyrifos, cypermethrin and chlordane were 1.4-, 1.5- and 1.3-fold greater, respectively, among workers held in groups compared with those held individually after insecticide treatment (Table 1). A more consistent dose response was observed when termites were held individually in microplate wells (Fig 1). Note that, for all insecticides examined, 100% mortality occurred at lower doses among termites held in groups than with those held individually. The microplate/individual bioassay method also provided more consistent responses for each replicate dose (Fig 1) than obtained with workers held in groups.

Experiments were conducted to examine whether trophallaxis, cannibalism or insecticide residue exposure contributed to the enhanced toxicity observed among termites held in groups after topical insecticide treatment (Table 2). This was achieved by using a mixture of dyed and undyed workers held together in groups of increasing ratio. Dye was used to distinguish insecticide-treated workers from those not receiving insecticide treatment. When dyed workers were treated topically with chlordane and placed immediately with untreated workers, significantly greater numbers of untreated workers were killed 24h after treatment compared with the control at all ratios examined. One hundred per cent mortality (at 24h) was observed among treated and untreated workers at all ratios except the lowest (one dyed-treated: nine undyed-untreated). These data indicated that workers topically treated with insecticide were capable of somehow transferring a lethal dose to untreated workers confined in the vial.

To examine whether insecticide was transferred by exposure to residue found on the chlordane-treated termites, experiments were conducted using dyed, chlordane-treated workers after they had been killed completely by chlordane topical exposure (LD_{99} dose, 48-h exposure, absence of any movement). Dyed,

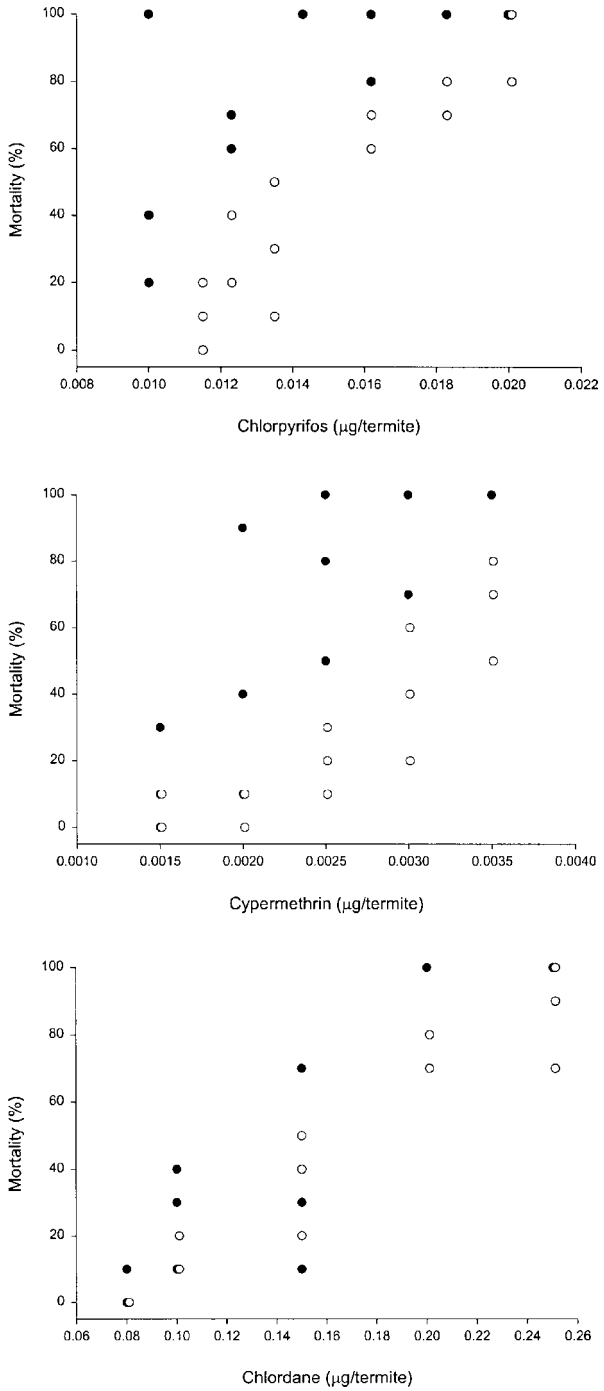

Figure 1. Mortality among *Coptotermes formosanus* workers treated topically with insecticide and subsequently confined (●) in groups (vials) or (○) individually (microplate wells). Each point represents an initial sample size of 10 termites.

Table 2. Mortality of *Coptotermes formosanus* workers when confined together under different treatment regimes

Ratio[b] dye-treated: untreated	Mortality (%) (±SE) for treatment[a]						Mortality (%) (±SE) for control[f]		
	LD_{99} (alive)[c]		LD_{99} (killed)[d]		Crushed[e]		Positive	Negative	
	Insecticide-treated	Untreated	Insecticide-treated	Untreated	Insecticide-treated	Untreated	Insecticide-treated	No insecticide treatment	
1:9	67 (±58)	7 (±6)	100	4 (±6)	100	0	100	0	0
2:8	100	100*	100	0	100	0	100	0	0
3:7	100	100*	100	5 (±8)	100	0	100	0	0
4:6	100	100*	100	0	100	6 (±10)	100	0	6 (±10)
5:5	100	100*	100	0	100	0	100	0	0
6:4	100	100*	100	0	100	8 (±14)	100	0	0
7:3	100	100*	100	0	100	0	100	0	0
8:2	100	100*	100	0	100	0	100	0	0
9:1	100	100*	100	33 (±58)	100	33 (±58)	100	0	0

[a] Three replicates were conducted. Asterisk indicates significant difference from respective controls (acetone alone) by Mann-Whitney test ($P < 0.05$). Only dyed termites received insecticide treatment.

[b] Ratio of dyed and untreated to undyed and treated termites.

[c] Dyed termites were treated with chlordane (LD_{99}) and placed immediately with untreated termites.

[d] Dyed termites were treated with chlordane (LD_{99}) and allowed to die (absence of movement) before being placed with untreated termites.

[e] Dyed termites were physically killed by having their heads crushed with forceps (no insecticide).

[f] Only dyed termites were treated with insecticide for the positive control. Termites in the negative control were not treated with insecticide.

chlordane-killed workers were placed with untreated workers in an increasing ratio as described previously (Table 2). Under these conditions, mortality among the untreated, undyed workers was not significantly different from the negative control (acetone treatment only), regardless of ratio. These data indicated that chlordane was not being transferred to the untreated termites in lethal quantities through residue contact or movement, even at very high treated-to-untreated ratios (Table 2). Further, the data suggest that trophallaxis or grooming may be the mechanism(s) by which insecticide is transferred to untreated workers in a lethal dose. The treated workers had to be alive at the beginning of the study for death to result in untreated workers. Alternatively, untreated workers may have avoided the insecticide-treated workers that were dead at the beginning of the experiment. Su *et al*[5] have reported that dead workers have a repellent effect on remaining colony members in *Coptotermes formosanus*.

The last experiment used dyed workers that had been physically killed by having their heads crushed with forceps (no insecticide was used). These killed workers were then placed with undyed, untreated workers and mortality was recorded 24h later. We hypothesized that at high ratios of dead to live workers, perhaps microbial release and/or growth on the corpses could cause mortality among the untreated workers in a group, as opposed to insecticide exposure. However, as with the previous experiment, mortality among the untreated group was not significantly different from the control, even at high killed-to-live ratios. Therefore, non-insecticidal factors, such as microbial growth, did not appear to be contributing to the mortality of workers.

This series of experiments demonstrated that untreated workers confined with workers that had been treated topically with chlordane received a lethal dose of insecticide. Transfer could have occurred by way of ingestion, either through trophallaxis and/or grooming behavior, or simply by passive uptake via residue exposure. Often, insecticides are more toxic by ingestion[19–21] than by topical exposure. In insects, topically applied insecticide rapidly covers the entire cuticular surface, including the foregut and hindgut. Because termites exhibit trophallaxis, it is very likely that the insecticide was being transferred in this fashion. Our reasoning for this supposition stems from the fact that untreated workers exposed to chlordane-treated workers died only when the treated workers were alive at the beginning of the experiment. Furthermore, the dose (LD_{99}) used against the workers represented a finite quantity of toxicant, presumably with the capacity to kill only a limited number of workers by topical application. Hence ingestion (if a more toxic route of exposure) would have increased the apparent toxicity of the limited insecticide dose.

Finally, experiments were conducted to demonstrate the exchange of insecticide among a group of chlordane-treated and untreated workers (Table 3). Five dyed, chlordane-treated workers were placed in a vial containing five untreated workers. No mortality was observed in the controls. As in the experiments described previously, mortality was observed in untreated workers confined with workers that had been topically treated with chlordane (Table 3). Mortality in the untreated worker group increased as the quantity of chlordane applied to the dyed workers increased. It is interesting that mortality among the chlordane-treated groups confined with untreated workers was lower at each corresponding dose compared with chlordane-treated workers maintained without any additional workers. These data indicated that topically applied chlordane was dispersed to

Dose[a]	Ratio[b] Dyed-treated:untreated	Mean mortality at 24 h (%) (±SD) Dyed-treated	Untreated	ng Chlordane per termite[c] Survivors	Moribund
0	5:0	0	NA	NA	NA
0	5:5	0	0	NA	NA
LD_{25}	5:0	80 (±28)	NA	NA	NA
LD_{50}	5:0	95 (±10)	NA	NA	NA
LD_{75}	5:0	100	NA	NA	NA
LD_{99}	5:0	100	NA	NA	NA
LD_{25}	5:5	50 (±58)	35 (±41)	8.4 (±0.8)	26.2 (±4.2)*
LD_{50}	5:5	70 (±26)	45 (±38)	11.8 (±6.3)	42.4 (±19.9)*
LD_{75}	5:5	95 (±10)	75 (±50)	25.1[d]	26.1 (±1.9)
LD_{99}	5:5	95 (±10)	100	0	32.3 (±11.5)

[a] LD_{25} = 0.13, LD_{50} = 0.16, LD_{75} = 0.21, LD_{99} = 0.4 μg per termite; lethal dose values were derived from bioassays of individually-held termites.

[b] Four replicates were conducted each comprised of five dyed, chlordane-treated workers and five or no untreated workers.

[c] Extractions of surviving and killed untreated termites.

[d] No variation because only one group survived the treatment.

NA, not applicable.

* Indicates significant difference ($P < 0.05$) from surviving termites by Students t-test.

Table 3. Mortality and chlordane recovery from *Coptotermes formosanus* workers placed together with an equal number of chlordane-treated Nile Blue A-dyed workers

untreated and chlordane-treated termites alike. Indeed, chlordane was recovered from untreated workers which had been confined with chlordane-treated workers (Table 3). In addition, significantly higher quantities of chlordane were recovered from dead workers exposed to chlordane-treated workers than from surviving workers exposed to chlordane-treated workers (Table 3).

Transfer of ingested toxicants or topical dusts from exposed to unexposed colony members has been attributed to trophallaxis and grooming behavior in two termite families.[3,9,11,22,23] Myles[9] successfully killed untreated workers with resin-coated, sulfluramid-treated workers by exploiting the trophallaxis behavior of termites. Although not definitive, our results suggest that lethal transfer of insecticide from workers treated topically with unformulated (active ingredient only) insecticide to untreated nestmates probably occurs by either trophallaxis or grooming, or a combination of these behaviors. Regardless of the mode of transfer, the exchange of insecticide significantly affects the outcome of toxicity evaluations. Insecticide bioassays conducted with worker termites confined individually after treatment provides a more accurate assessment of toxicity among individual termites. Our data indicated that the apparent toxicity of a pesticide is artificially elevated when termite workers are held in groups. The microplate/individual assay we describe is a facile, accurate and inexpensive method for determining insecticide toxicity in insects that exhibit trophallactic and grooming behaviors.

ACKNOWLEDGEMENTS

We thank CA Strong and JD Hall for technical assistance and Drs SJ Yu (University of Florida) and D Oi (USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology) for reviewing the manuscript.

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